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(71) Anmelder:

Balazs, Viktor, Dr. Dr.med., 5068 Odenthal, DE

(74) Vertreter:

Bauer, W., Dipl.-Phys. Dr.rer.nat., Pat.-Anw., 5000 Köln

(72) Erfinder:

Balazs, Viktor, Dr. Dr.med.; Balazs-Fröhlich, Margit, Dr.rer.nat., 5068 Odenthal, DE

(54) Verfahren zur Untersuchung einer azellularen biologischen Flüssigkeit auf zellulare Onkogen-Transkripte bzw. deren Fragmente

Bei dem Verfahren zur Untersuchung einer azellularen biologischen Flüssigkeit auf die Anwesenheit von zellularen Onkogen-(RNA)-Transkripten, bzw. deren Fragmente wird
a) die azellulare biologische Flüssigkeit (vorzugsweise Blutplasma) so behandelt, daß die RNA in Anwesenheit eines RNase Hemmers getrennt auf einem festen Träger in denaturierter Form immobilisiert ist,
b) der feste Träger mit markierten Onkogen-DNA Proben in Kontakt gebracht, um diese mit der RNA zu hybridisieren, wenn komplementäre Sequenz vorliegt, und
c) das Produkt auf die Anwesenheit markierter DNA bestimmt.

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(71) Applicant:
Dr. Viktor Balazs, M.D.
5068 Odenthal, DE

(72) Inventor:
Dr. Viktor Balazs, M.D.;
Margit Balazs-Fröhlich,
Dr.rer.nat., 5068 Odenthal, DE

(74) Representative:
W. Bauer, Dipl.-Phys. Dr.rer.nat.,
Patent Attorney,
5000 Köln

(54) Method for the investigation of an acellular biological fluid for cellular oncogen transcripts and/or their fragments

In the method for the investigation of an acellular biological fluid for the presence of cellular oncogen-(RNA)-transcripts and/or their fragments,

a) the acellular biological fluid (preferably blood plasma) is treated so that, in the presence of an RNase inhibitor, the RNA is separately immobilized on a solid carrier in the denatured form,

b) the solid carrier is brought into contact with marked oncogen-DNA samples in order to hybridize these with the RNA when a complementary sequence is present, and

c) the product is analyzed for the presence of marked DNA.

Patent Claims

1. Method for the investigation of an acellular biological fluid for the presence of cellular oncogen-(RNA)-transcripts and/or their fragments, by

a) separating the RNA from the acellular biological fluid and immobilizing the RNA on a solid carrier in the denatured form,
b) bringing the solid carrier in contact with marked oncogen-DNA samples in order to hybridize these with the RNA, when a complementary sequence is present, and
c) analyzing the product for the presence of marked DNA.

2. Method according to Claim 1, characterized by the fact that the acellular biological fluid is human blood plasma and that the separation of the RNA carried out according to a) is performed in the constant presence of an RNase inhibitor.

3. Method according to Claim 1 or 2, characterized by the fact that the sample is radioisotope-marked oncogen-DNA.

4. Method according to Claim 1 or 2, characterized by the fact that the sample is not radioisotope-marked, but preferably biotin-marked oncogen-DNA.

5. Method according to Claim 1 and 2, characterized by the fact that before the separation of the RNA and the immobilization of the RNA on the solid carrier, the blood plasma is treated with an organic solvent in order to remove protein material in an organic phase and in order to obtain the RNA in the aqueous phase.

6. Method according to Claims 3 and 5, characterized by the fact that the determination of the presence of marked DNA is carried out with an autoradiographic technique.

7. Method according to Claims 4 and 5, characterized by the fact that the determination of the presence of marked RNA is carried out with an enzyme affinity test using a color reaction.

8. Method according to Claim 5, characterized by the fact that pure nitrocellulose is used as solid substrate.

9. Method according to Claim 5, characterized by the fact that vacuum is used for the immobilization.

10. Method according to Claim 2, characterized by the fact that

a) before step a), the blood is mixed with a solution of an effective RNase-enzyme-inhibitory substance and that the plasma is separated;
b) the product of step a) (the plasma) is brought into contact with a detergent and a proteolytic enzyme;
c) the product of step b) is mixed and treated with a protein-denaturing solution, such as guanidinium isocyanate;
d) the product of step c) is extracted with an organic solvent (at 60°C) with the formation of an essentially protein-free aqueous solution, which contains the RNA;
e) the product of step d) is brought into contact with a detergent and a proteolytic enzyme, incubated and then the extraction of step d) is repeated;
f) the product of step e) is precipitated with ethanol (2 volumes), washed and dissolved in sterile water;
g) the product of step f) is subjected to a DNase enzyme treatment in order to degrade the DNA present and to remove it and then the extraction is step d) is repeated; or
h) the product of step f) is subjected to an affinity adsorption and elution method with oligo-(dT)-cellulose in order to isolate the poly(A)$^+$RNA (mRNA).

11. Method according to Claim 10, characterized by the fact that
a) the product of step g) or h) is treated and then denatured;
b) the product of step a) is applied onto a solid substrate and immobilized by baking in vacuum;
c) the solid substrate of step b) is brought into contact with a solution of marked oncogen-DNA sample in order to hybridize it with the RNA immobilized on the solid substrate;

d) the product of step c) is determined by autoradiography in case of radioisotope-marked oncogen-DNA sample;

e) in case of an oncogen-DNA sample which is not marked with a radioisotope, the product of step c) is determined by an enzyme affinity test using a color reaction.

12. Method according to Claim 11, characterized by the fact that the solid substrate of step b) is treated in order to prevent further binding of nucleic acid on it.

13. Method according to Claim 11, characterized by the fact that the solid substrate of step c) is washed in order to remove nonspecifically bound marked oncogen-DNA.

14. Method according to Claim 11, 12 or 13, characterized by the fact that the remaining radioactivity bound to the plasma-RNA is determined by autoradiography.

15. Method according to Claim 11, characterized by the fact that the remaining nonradioactive marking substance of the solid substrate, which is bound by hybridization between RNA and the marked oncogen-DNA, is determined by an enzyme affinity test using a color reaction.

16. Method according to one of Claims 1 to 14, characterized by the fact that the oncogen-DNA, which was hybridized with the RNA bound on the solid substrate, is removed, in order to further hybridize (rehybridize) the solid substrate having the RNA remaining on it, with a new oncogen-DNA sample.

17. Method according to one of Claims 1 to 16, characterized by the fact that an individual oncogen-DNA or a mixture of individual oncogen-DNAs is used as oncogen-DNA sample.

Specification

The invention is concerned with a method for the detection of cellular oncogen transcripts and/or their fragments, according to the main clause of Claim 1.

Methods for the determination of cellular oncogen-(RNA-) transcripts, including separation of RNA, mRNA, denaturing and hybridizing and similar are known in the field of molecular biology research. Reproducibility, reliability, specificity and extreme sensitivity of in-vitro hybridization between DNA and DNA, as well as between RNA and DNA, is known from the literature, and found application as standard method for the solution of specific problems.

Hybridization of RNA with DNA using a solid substrate for immobilization has been known since 1965 (J. Mol. Biol., 12: 829-942; 1965). Since then, several improvements of this method as well as of the methods of separation of the RNA from cells and tissues have been published.

The activation of two or more cellular oncogens by increased transcription or by mutation is generally considered to be a very important step in the development, maintenance and progression of malignant transformations. This increase of transcription can reach several times and even 100 times the normal values.

The following most important causes or circumstances can be regarded as decisively responsible for the elevated and altered RNA content of the blood plasma in malignant conditions:

1. Elevated transcription of two or more cellular oncogens (see above).
2. Increased cell turnover in malignant states.
3. Elevated secretion of RNA or poly(A)$^+$RNA (mRNA) by the living malignant cells, as experiments with in-vitro grown malignant cell cultures already showed.
4. Elevated mobilization of the RNA, especially mRNA, from the cell nucleus by cancer-specific products, such as "cancer-associated protein", etc.
5. Elevated RNA density in peripheral zones of malignant cells.

6. Elevated leakage of living malignant cells, but especially dying and necrotic malignant cells.
7. Increased death rate of malignant cells.
8. Increased vascularization of the malignant tumors.
9. Increased permeability of the capillaries and vessels in malignant tumors for macromolecules and cell components because of the unsuitable and incomplete development of the otherwise richly present vessels.
10. Accumulation of malignant cells, namely dying cells, in the capillaries and arterioles of malignant tumors, so that cell elements can arrive more easily directly into the bloodstream.
11. Elevated resistance of the RNA, mRNA and their fragments to enzymes, which degrade them, such as RNase because of:
    a) bonding on proteins, mucoproteins, lipoproteins or protection by a lipoprotein shell and
    b) presence of RNA or its fragments in the ds.RNA form (ds stands for double-stranded RNA).
12. Possible longer residence of RNA or its fragments in the bloodstream because of the exhaustion of the clearance mechanisms which would be responsible for the removal of RNA or its fragments, possibly because of the constant "flooding" of the bloodstream with these substances in malignant conditions or because of other, still unknown, reasons.

The above-mentioned causes and conditions are responsible for the elevated, altered RNA content in the blood plasma, acting in different combinations and to different degrees in the various types and stages of malignant conditions. The elevated transcription of cellular oncogens is the fundamental prerequisite but is not sufficient alone. In precancerous conditions, such as in colon polyps, for example, the transcription of one of the cellular oncogens may be increased even 90 times, but, in spite of this, no RNA transcripts of such highly activated oncogen appears in the blood plasma. Other and several of the conditions characteristic for malignant states must be present at the same time and work together.

In-vitro molecular hybridization is not only extremely specific, but also highly sensitive. Even 1 pg of RNA can be detected in a complex population of RNA molecules.

Since early recognition is the most important prerequisite for remission or healing of malignant diseases, a universal malignancy test is necessary which can sense or detect, in the broad spectrum of malignant diseases, even small amounts of malignant cells which cannot yet be detected with the diagnostic measures commonly used today.

Such a universal screening test is not available as yet. Only two methods have found routine application as progress control tests: the alpha-photo protein test for liver cancer and teratocarcinoma, and the carcinoembryonic antigen (CEA) for various cancers of the digestive system.

In 1985, a flotation method after 16-hour ultracentrifuging of the serum has become known, which can detect a lipoprotein fraction characteristic for cancer, this fraction also containing RNA with poly(A)$^+$RNA components. However, this method has not yet been tested in precancerous conditions, in beginning monoclonal gammapathies and in many other conditions. The sensitivity of this flotation method is relatively low, 0.2 micrograms/mL, in comparison to the highly sensitive hybridization method. This can probably explain why the flotation method gave negative results in some clinically confirmed cases of cancer. It is very probable that the RNA in the lipoprotein fraction is only a part of the total plasma-RNA which has arrived from the malignant cells into the bloodstream (total plasma-RNA of malignant origin). The long ultracentrifuging process is a great disadvantage of the flotation method.

Recently, two immune tests were also proposed as diagnostic cancer tests. A method for the isolation of the so-called cancer-associated protein (CAP) was published with the intent of using it as a cancer test (immune test).

In 1985, the detection of oncogen-polypeptides with monoclonal antibodies in the urine using immunoblot was considered as a possibility for a cancer test. Investigations carried out with polypeptides of three oncogens showed that the difference between normal and pathological values is not very large and that pregnancy produces higher values than a malignant disease. For complete evaluation, each oncogen-polypeptide must be investigated for various quantities, which is very time-consuming and uses a lot of material.

Furthermore, the normal values showed such a large scattering that a significant overlap between normal and pathological values may occur. For example, investigations with monoclonal antibodies against only one oncogen-polypeptide showed positive values in 25-29% of the malignant cases and in 6-9% of the nonmalignant cases.

A very significant disadvantage of this method and every other method which is based on the immunological detection of proteins or polypeptides of cancer cell origin is that the presence of specific antibodies, formed by the host or introduced for therapeutic purposes, causes the occurrence of unrealistic results and can be a significant perturbing factor.

The task of the invention is to provide a method for the detection of RNA transcripts or their fragments, of cellular oncogens in an acellular biological fluid, such as blood plasma. Since it is necessary for this purpose to first separate the RNA from the blood plasma, another task of the invention is to provide a reliable method for the separation of the RNA from the blood plasma.

This task is solved by a method according to Claim 1. By using reliable, effective and potent RNA inhibitors, by avoiding the mixing of the ubiquitous, highly resistant RNase enzyme from exogenous sources during the entire process, by treatment of the blood plasma for the separation of the RNA content, by immobilizing the RNA in the denatured form on a solid substrate and by contact of the solid substrate with the marked denatured oncogen-DNA in order to bind these together (to hybridize them) when the plasma-RNA and the oncogen-DNA have complementary sequence(s), a reliable detection method is achieved. This fact is determined by the detection of the marking substance or, in the case of radioisotope marking, by autoradiography.

According to the invention, the total plasma-RNA as well as the RNA which reach the bloodstream, mRNA or their fragments are isolated from the blood plasma of patients with malignant diseases and are investigated for the presence of the RNA transcripts of cellular oncogens using in-vitro molecular hybridization.

The practical applicability of the method for the determination of RNA transcripts or of their fragments, of cellular oncogens from human blood plasma, is a universal malignancy test for

the detection of malignant processes (as confirmation test) for early recognition of malignancy (as a screening test) and for early detection of relapses and metastases after therapeutic measures (as an ongoing control test).

According to the invention, a characteristic or a component for blood plasma-RNA was found which can differentiate between malignant or nonmalignant origin. The messenger activity of RNA, tested in cell-free protein-synthesizing systems was found to be unsuitable for this, because this activity could be observed both in malignant and nonmalignant states. However, such differentiation could be achieved due to the presence of oncogen transcripts or their fragments in the plasma-RNA. Even in the first blind experimental series, in malignant cases, oncogen transcripts or their fragments were detected in all plasma-Ranks, while they were not detected in any of plasma-Ranks from nonmalignant states. Further investigations confirm this tendency and indicate that this method is able to recognize smaller amounts of malignant cells, which are not detectable today using the usual diagnostic methods.

The essential advantage of this invention is that it provides a method which can be used as a universal malignancy test: this test is based on the basic principles of malignant transformation by the activation of cellular oncogens and on the special characteristics of malignant cells and tumors.

The second significant advantage of this method is its high sensitivity (1-5 pg/mL).

In contrast to immune tests, another essential advantage of the invention is that the specific antibodies, which were formed by the host or were introduced into the bloodstream for therapy purposes, cannot influence the results.

In contrast to the detection methods which are based on immunological detection of proteins or polypeptides of cancer cell origin, the method according to the invention has the advantage that perturbation of the results by specific antibodies does not occur.

The detection of cellular oncogen transcripts or their fragments from the blood plasma provides additional information that is relevant to therapy beyond the actual malignancy test:

1. a) The basic characteristic of malignant processes and their behavior in the host is essentially determined by the fact as to which oncogens are activated (genetic scripture cell oncogen profile). So far, this could only be determined histologically in tumor tissues by in-vitro nucleic acid hybridization. The malignancy test according to the invention can provide this information from the blood plasma, at least partly (plasma oncogen profile) long before the relatively small tumor cell mass can be visualized and can be available for histological investigations.

1. b) During the malignant processes in vitro as well as during the existence of malignant disease in patients, new oncogens can be activated additionally, which can lead to a significant progression of the malignancy. This danger can be recognized early with the malignancy test during process control by the fact that a new oncogen transcript will be identified in the blood plasma, which has not been present in the plasma previously.

1. c) During the course of the disease, an amplification of the activated oncogens may occur, which can cause increased aggressiveness and progression of the malignant cells. The occurrence of such amplification can be observed with the quantitatively performed malignancy test early during progress control. This means that a subclone of the tumor cells, which has the amplified oncogen, can be recognized early before it can spread and could make the prognosis very much worse. This subclone can be influenced in the early stages by specific immune therapy or immunochemotherapy and possibly destroyed. Amplification of cellular oncogens (by two to one hundred times) occurs either in the primary tumor or during the progression and diversification of malignant cells and occurs in approximately 30% to 50% of malignant diseases.

2. Gene products of the activated cellular oncogens play an important, an even vital function in the transformed malignant cells, and many of these function as protein kinase enzymes in the cell membrane. The change of or adverse effect on this function by attacking the malignant cells immunospecifically, targeted on their oncogen products with monoclonal antibodies alone or bound to radioactively or chemotherapeutically acting substances, can lead to a healing action before the tumor cell mass can spread. In early recognition, the

plasma oncogen profile shows the specific therapeutic possibilities. The control of progress with new oncogen samples makes it possible to recognize newly occurring oncogen transcripts. A quantitatively performed malignancy test provides during the progress of the disease early detection of possible amplification of a cellular oncogen and gives specific information how this new subclone of malignant cells can be suppressed or destroyed before greater progression or aggressiveness is produced.

Below, the invention is explained in more detail. The freshly obtained blood is mixed immediately with an RNase-enzyme-inhibiting substance and the plasma is separated rapidly. Then the blood plasma, in the presence of an RNase inhibitor, is mixed with a detergent, such as sodium dodecylsulfate (SDS) and a proteolytic enzyme, such as Proteinase K (Boehringer, Mannheim) in an aqueous medium and then it is incubated at 37°C for 60 minutes in a buffer solution (pH 7.5). Then it is mixed with 4 M guanidinium isothiocyanate in order to denature the protein and cleave off the proteins from the RNA. Then the mixture is extracted with organic solvents, such as phenol and chloroform at 60°C in order to remove the majority of the proteins from the organic phase, which leads to an aqueous phase which essentially contains the entire amount of RNA. This aqueous phase is extracted with phenol/chloroform again. Then it is extracted twice with chloroform. The RNA is precipitated by mixing with two volumes of ethanol at -20°C. The RNA is dissolved in Tris-Cl buffer (pH 7.4) with EDTA and SDS and treated with Proteinase K at 37°C for one hour in the presence of RNase inhibitors. Then it is extracted twice with chloroform at 60°C. It is extracted with chloroform twice at room temperature, the RNA is precipitated with ethanol, washed and stored in 70% ethanol at -70°C.

Either the poly(A)$^+$RNA is selected from the RNA by affinity adsorption and elution methods with oligo (dT) cellulose, or the RNA is subjected to a DNase enzyme treatment in order to degrade and remove any DNA present.

The denatured RNA or poly(A)$^+$RNA is applied onto pure nitrocellulose paper as a solid substrate and, in order to immobilize the RNA on the solid substrate, it is treated for 2 hours at 80°C in a vacuum oven. The baked solid substrate is then treated in order to prevent further binding of nucleic acids to the solid substrate.

An amount of purified molecularly cloned DNA, which contains the sequence for the oncogen as code, is marked either with a radioisotope or not. Radioisotope marking is carried out by Nick Translation according to Rigby et al. (J. Mol. Biol. 113: 237-251, 1977) in order to reach high specific activities. The radioisotopes that can be used for this method are especially 32^{P} [should be P^{32} - Translator], but also I^{125}, I^{131} and H^{3}.

For nonradioisotope marking of the oncogen-DNA samples, for example, biotin is used (Proc. Natl. Acad. Sci. USA 78: 6633-6637, 1981) and, more recently, photobiotin (Nucleic Acid Research 13: 745-761, 1985). The marking with photobiotin is especially uncomplicated, rapid and cost-effective. A photobiotin marking and detection kit is offered commercially (BRESA, Adelaide, South Australia).

Oncogen-DNA samples, marked or unmarked, are available commercially (ONCOR, Inc., Gaithersburgh, MA, USA 20877; ONCOGENE SCIENCE Inc., Mineola, NY, 11501 USA).

The marked oncogen-DNA sample is denatured and is brought in contact with the solid substrate on which the plasma-RNA is immobilized, in order to hybridize it with it Thus the marked oncogen-DNA sample will be bonded (hybridized) through hydrogen bonds with the complementary sequence(s) of the plasma-RNA, which is immobilized on the solid substrate.

After a predetermined time period, the solid substrate is subjected to washing, in order to remove nonspecifically bound marked oncogen-DNA samples.

The solid substrate is then subjected to analysis in order to detect the occurrence of hybridization between the plasma RNA and the marked oncogen-DNA sample. In the case of radioisotope marking, this is carried out by autoradiography, and in case of biotin marking, the detection of biotin using an enzyme affinity test with a color reaction.

The following example serves to further explain the invention, without representing a limitation of it.

Example

Twenty mL of blood with 20 IU/mL of heparin is taken and is immediately mixed with a solution of an RNase enzyme inhibitor such as RNasin (Promega Biotec, Madison, WI, USA) (end concentration: 2000 U/mL or with a vanadyl ribonucleoside complex (Bethesda Research Lab., Gaithersburg, MD, USA) (end concentration: 10 mM). The blood plasma is separated as rapidly as possible. Five mL of blood plasma is pipetted in each of two disposable plastic centrifuge tubes, one of which is stored deep-frozen for any repetition or additional investigations as reserve. The other is processed immediately.

A mixture of 0.2 M Tris Cl (pH 7.5); 25 mM ethylenediaminetetraacetic acid (EDTA); 0.3 M NaCl; 2% (weight/volume) of sodium dodecylsulfate (SDS) and Proteinase K (end concentration of 200 micrograms/mL) are added and incubated in the presence of RNase inhibitors at 37°C for 60 minutes. Then it is mixed with 4 M guanidinium isothiocyanate. The slimy mixture is drawn at 60°C into a syringe which is equipped with an 18 g needle and is ejected again forcefully until the viscosity is considerably reduced. Then the same volume of phenol and chloroform/isoamyl alcohol (24:1) is added and extraction is performed at 60°C for 10 to 15 minutes by vigorous shaking. After centrifuging, the aqueous phase is extracted twice again with phenol-chloroform and then twice with chloroform. The aqueous phase is mixed with 2 volumes of ethanol and precipitated at -20°C for 1 to 2 hours as RNA.

After centrifuging, the RNA is dissolved in Tris.-Cl (0.1 M, pH 7.4) buffer solution, which also contains 50 mM of NaCl, 10 mM of EDTA, 2% SDS and RNase inhibitor, and incubated with Proteinase K (end concentration 200 micrograms/mL) at 37°C for 1 h. Then it is extracted twice with phenol and chloroform at 60°C and with chloroform at room temperature, again twice. The RNA is precipitated from the aqueous phase with ethanol, washed with 70% ethanol and dissolved in the necessary solution.

The extraction of the RNA is essentially and significantly facilitated by the use of the instrument "Nucleic Acid Extractor" (Applied Biosystem, Foster City, CA, USA). The method described above can be easily adapted to this instrument.

Either the poly(A)$^+$RNA is selected from the isolated plasma-RNA with oligo-(dT) cellulose using batch absorption and elution, or the plasma-RNA is subjected to a DNase enzyme treatment in order to degrade any DNA present.

For the selection of the poly(A)$^+$RNA, the plasma-RNA is dissolved in sterile water, incubated at 65°C for 10 minutes and mixed with 2 x loading buffer and cooled to room temperature (loading buffer: 20 mM Tris.Cl, pH 7.6: 0.5 M NaCl; 1 mM EDTA; 0.1% SDS). Then it is mixed with 0.3 g (dry weight) of oligo-(dT) cellulose for each 0.5 mg of RNA, centrifuged at 1500 g for 4 minutes at 15°C and the oligo-(dT) cellulose is washed 4-5 times with 5 mL of loading buffer (at room temperature). The poly(A)$^+$RNA is subjected to elution with 1 mL washing of sterile 10 mM Tris. Cl (pH 7.5);1 mM EDTA; 0.05% SDS.

Na acetate (3 M pH 5.2) is mixed to obtain 0.3 M, the RNA is precipitated with 2.2 volume of ethanol at -20°C and the precipitate is washed with 70% ethanol.

When the selection of the poly(A)$^+$RNA is not performed, the plasma-RNA is subjected to a DNase enzyme treatment. In this case, the RNase-free DNase enzyme and $MgCl_2$ (to 2 mM) are mixed in the plasma-RNA solution (50 mM Tris.Cl, pH 7.5 in 1 mM EDTA) and incubated for 30 minutes at 37°C in the presence of RNase inhibitor, then is extracted with phenol/chloroform and the RNA is precipitated in the presence of Na acetate (pH 5.2, 0.3 M) with 70% ethanol at -70°C.

The following well-known commercially available 18 molecularly cloned human oncogens were used as oncogen-DNA sample, which are here grouped approximately according to the frequency of their occurrence with elevated activity in human malignancies:

in all or practically all malignancies: A	rarely, only in certain forms of malignancies: B		in very rare forms of malignancies: C	
fos	fes	raf	Erb^A	alu
myc	myb	Erb^B	mos	bcr
Ha-ras	fms	N-myc	sis	N-ras
Ki-ras	src			
	abl			

The samples were obtained either marked with P^{32} radioisotopically, (specific activity: 10^8 cpm/microgram) by Nick Translation according to Rigby et al. (J. Mol. Biol. 133: 237-251, 1977) or were unmarked. The unmarked DNA samples were marked either with biotin (according to Leary, see page 21) or with photobiotin according to the directions of the manufacturer (BRESA, Adelaide, South Australia): after short irradiation with visible light, photobiotin goes into a stable bond with nucleic acids. The DNA marked with biotin in this way can be isolated with 2-butanol extraction and ethanol precipitation and can be stored as stable marked sample for half a year in 0.1 mM EDTA at -15°C in a stable manner.

The plasma-RNA or the poly(A)$^+$RNA is subjected before hybridization to a treatment called prehybridization. The RNA solution or RNA dilutions are denatured first by incubation at 65°C for 15 minutes and then rapid cooling. Then the RNA is applied onto the solid substrate, such as nitrocellulose paper, which was first equilibrated with 20 x NaCl/Cit and then dried.

A corresponding volume of the denatured RNA solution is introduced into a well of the microfilter plate (Bio-Dot Microfiltration Apparatus, Bio-Rad, Richmond, CA., 94804 USA; Minifold Filtration & Incubation Plate, Schleicher & Schuell, Keene, New Hampshire, 03431 USA) and is filtered under gentle vacuum with the formation of a spot of 3.0 mm diameter. The quantity of the applied RNA: 10 micrograms, of the poly(A)$^+$RNA: 2 micrograms.

The nitrocellulose paper is then dried for 2 h at 80°C in a vacuum oven. After baking, it is incubated in a solution (prehybridization buffer): formamide (50% volume/volume);

5 x NaCl/Cit; 50 mM sodium phosphate, pH 6.5; sonicated, denatured salmon sperm DNA (250 micrograms/mL) and 0.02% of each of bovine serum albumin (BSA), Ficoll and polyvinylpyrrolidone for 8-20 h at 42°C.

The radioisotope-marked human oncogen-DNA sample (2 x 10^6 cpm/mL) is first denatured at 100°C for 5-10 minutes, cooled rapidly and is mixed in the solution (prehybridization buffer), which contains the nitrocellulose paper with the immobilized plasma-RNA. The hybridization is carried out at 42°C for 20 hours. Then the nitrocellulose paper is washed with a solution of 2 x NaCl/Cit, 0.1% SDS, first at room temperature and then at 50°C several times with the solution of 0.1x NaCl/Cit, 0.1% SDS.

The nitrocellulose paper is placed between clear acetate films and brought into the neighborhood of film which is sensitive to x-rays. An amplifier screen is applied on the opposite side and packed so that it is light-tight. After a certain period of time, the film was developed. The presence of the RNA transcripts or of their fragments, which hybridized with the oncogen-DNA sample, is determined by the presence of a spot on the film.

In the case of nonradioisotope-marked oncogen-DNA samples, such as in the case of oncogen-DNA samples marked with photobiotin, the DNA samples are introduced into 0.1 M EDTA, otherwise, the prehybridization is carried out in the same way as with the radioisotope-marked DNA samples, but the duration of the prehybridization is shorter (4-8 h). The hybridization is carried out as with the radioactive samples, but at a higher temperature (55°C) for 20 h in a solution of: 4 volume prehybridization buffer; 1 volume of approximately 0.5 g/mL of sodium dextransulfate and 20 ng/mL biotin-marked DNA sample (oncogen-DNA sample).

The dried nitrocellulose papers are incubated at 42°C for 30 minutes in STMT buffer (1 M NaCl; Tris. Cl, pH 7.5; 2 mM $MgCl_2$ 0.05% v/v Triton X-100) with 30 mg of bovine serum albumin. After the nitrocellulose paper has been dried, it is brought in contact with 1 microgram/mL Sigma avidin-alkaline phosphatase complex at room temperature for 10 minutes, and then it is incubated under frequent shaking in an STMT buffer (3 x 10 min) and STMT buffer (1 m NaCl, Tris. Cl, pH 9.5, 5 mM $MgCl_2$) for 2 x 5 minutes.

For the color reactions, the nitrocellulose paper is mixed at room temperature in the dark with substrate solution (STM buffer, but only with 0.1 M NaCl) with 0.33 mg/mL nitro-blue-tetrazolium, 0.17 mg/mL 50-bromo-4-chloro-3-indolyl phosphate and 0.33% v/v N,N-dimethylformamide. The reaction is ended by washing the nitrocellulose paper with 10 mM Tris.Cl, pH 7.5; 1 mM EDTA.

The detection for the presence of oncogen-RNA transcripts or of their fragments is positive, when the plasma-RNA or the poly(A)$^+$RNA gives a positive signal (above the background or above the negative control) with at least one of the oncogen-DNA samples. In the case of the radioactive sample, a darker spot appears on the film after autoradiography; in the case of biotin-marked samples a spot with a color reaction appears. The malignancy test is negative when no RNA can be isolated from the blood plasma, or when the blood plasma RNA does not give a positive signal with any of the oncogen samples.

The reuse of the blood plasma RNA, which is immobilized on nitrocellulose paper, is possible after hybridization and autoradiography (for rehybridization). The hybridized DNA sample can be removed by washing at 65°C for 1-2 h with 0.1 to 0.05 x washing buffer (1 x washing buffer: 50 mM Tris. Cl, pH 8.0; 0.2 mM EDTA; 0.5% sodium pyrophosphate and 0.02% of each of BSA, Ficoll, polyvinylpyrrolidone). The treatment of the nitrocellulose paper, that is, the prehybridization as well as rehybridization is carried out as above (original). In this way, the rehybridization with new oncogen-DNA samples can be performed. This is very important, because the amount of blood plasma RNA is very limited.

The mode of operation according to the invention for the detection of RNA transcripts of their fragments, of cellular oncogens in human blood plasma, includes several favorable reaction methods and steps in order to give high reliability and reproducibility. Thus, the use of reliable RNase inhibitory substances permits prevention of any degradation of RNA, even after taking the blood samples. The danger of mixing (admixing) of the ubiquitous high-grade resistant RNase from exogenous sources is reduced during the entire process of the method by the use of baked glassware autoclave solutions, baked spatulas, tools, dry chemical preparations, of autoclave sterilized water that was distilled in glass, as well as wearing gloves during all phases and steps of the preparation and investigation.

The use of proteinase K facilitates the removal of proteins, the guanidinium isothiocyanate treatment facilitates the denaturing of the proteins as well as the cleavage of the RNA protein complexes. The rest of the proteins is removed by organic extraction. The degradation and the removal of the DNA is achieved by the DNase treatment because otherwise the DNA could interfere by hybridization. Baking ensures the reliability of the solid bond of RNA to the nitrocellulose filter. The treatment of the nitrocellulose filter after baking prevents nonspecific compounds and also ensures reliability. The washing of the nitrocellulose filter after hybridization removes superfluous or nonspecifically-bound, marked oncogen-DNA samples and also contributes to reliability.

Numerous modifications are possible.
A quantitative evaluation of the positive test is possible.

A semiquantitative evaluation can also be carried out. In this case, the intensity of the black spot on the x-ray film is measured using densitometry and quantitative comparisons can be made (Reflectance Densitometer, Bio-Rad, Richmond CA 94804, USA).

Quantitative Test

When a blood plasma-RNA shows a positive test with an oncogen sample, the relative amounts can be determined by the dilution method. In this case, a 1:2 dilution series is prepared from the blood plasma-RNA and each dilution is tested by hybridization. The highest dilution which still gives a positive signal is the titer. Thus, in routine control of a patient during the observation time, quantitative comparisons can be made. In this way, for example, the amplification of an activated oncogen can be observed: when, during the routine control of the titer of an oncogen is increased not in proportion, in comparison to other oncogens, this means the amplification of this oncogen.

If one uses high sensitivity, such as in the screening of persons who are especially susceptible to cancer due to familial, genetic reasons or because of occupational exposure, or in the screening of patients who, because of chemotherapy or immunosuppressant therapy, have a

compromised immune system, radioactive oncogen samples with high specificity can be used or, according to Leary et al. (Proc. Nat. Acad. Sci. USA 80: 4045-4049, 1983), biotin-marked oncogen samples and enzyme affinity test can be used and these - when possible - hybridized with the poly(A)$^+$RNA selected from the plasma-RNA. Thus, a sensitivity of 1 pg/mL of RNA can be achieved. Otherwise, the use of the plasma-RNA for hybridization is sufficient for differential diagnostic purposes (as a confirmation test) for running control, monitoring and staging.

In general, a positive test is obtained in the case of a malignant disease, mostly when the plasma-RNA was first hybridized only with oncogen group A (see above) because these oncogens are activated in almost all types of malignancies. Hybridization is more rarely extended to group B.

If the type of cancer is known (in running control) or there is a suspicion of a certain type of cancer, oncogens can be used as samples which are especially frequently activated in this type of cancer. New oncogens are being discovered continuously. Sometimes these can also be used as samples.

However, if one wishes to know which activated oncogens are present in the bloodstream (plasma oncogen profile), the blood plasma-RNA must be hybridized with all oncogens of groups A and B and rarely of group C. The same has to be done when the appearance of new oncogens would have to be detected in the bloodstream during the course of the disease, which is of prognostic and therapeutic importance.